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Expression of pro-urokinase cDNA in Chinese hamster ovary cell line.

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Thank you,

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Expression of Pro-urokinase cDNA in Chinese Hamster Ovary Cell Line

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Expression vectors containing the pro-urokinase (pro-UK) cDNA (pSV_2-proUK) and a dihydrofolate reductase cDNA (pSV_2-dhfr or MMTV-dhfr) were cotransfected into CHO-dhfr$^-$ cells by the calcium phosphate precipitation technique. The dhfr$^+$ transformants were selected by fibrinolytic agarose plate assay. Two colonies, named CLF-14 and CLF-8, exhibited significantly high expression levels of the biological activity of urokinase-type plasminogen activitor (*u*—Pa). They reached more than 24IU/10^6 cells/48h and 16IU/10^6 cells/48h, respectively. Examination of the cell supernatants for *u*—Pa antigenicity using ELISA method also showed strong positive results, and the quantities of expression were about 0.14-0.22 μg/10^6 cells/48h and 0.08-0.14 μg/10^6 cells/48h, respectively. The *u*—Pa secreted by stable transformed cells could be completely inhibited by UK anti-serum, but not by tissue-type plasminogen activator (*t*—PA) antiserum nor by normal rabbit serum.

KEY WORDS: Prourokinase cDNA; CHO-dhfr$^-$ cell; gene expression

INTRODUCTION

Prourokinase, also called single chain urokinase, is a promising new type of thrombolytic agent which has greater affinity to blood clot than urokinase. Prourokinase as a pharmaceutical drug has low side effects and causes only slight systematic bleeding clinically. However, it is difficult to obtain prourokinase from nature (e.g. there is only 0.1 percent of urokinase in urine). Perhaps the best way to produce pharmaceutical prourokinase would be to rely on the technique of genetic engineering. A full length cDNA of prourokinase has been reported (Holmes, *et al.* 1985)[1], and additional literature about prourokinase have been published (Jacobs *et al.* 1985; Cheng *et al.* 1988; Klein *et*

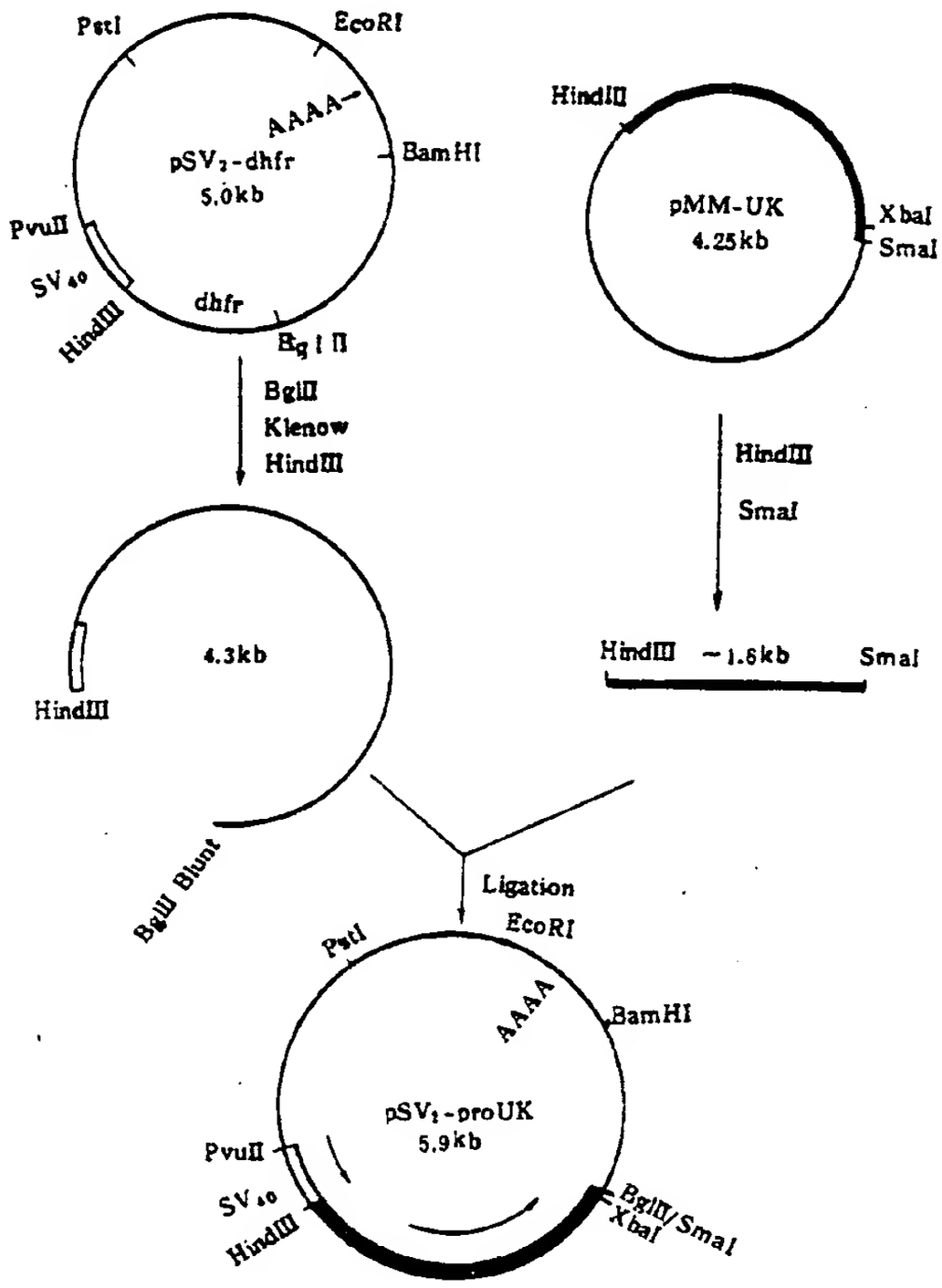

Fig. 1
Construction of transfer vector pSV_2—proUK.
⁻ - Sequence of SV40 early promoter
■ - Sequence of pro-UK cDNA
AAAA→ - Sequence of poly A signal

al. 1988; Pierard *et al*. 1989; Winkler *et al*. 1985) [2-6]. Recently, a full length prourokinase cDNA coding for both the 411 amino acid residues of structural gene and the 20 amino acid residues of signal peptide was obtained in our laboratory by reverse transcription technique and identified by restriction mapping and DNA sequencing analysis (Fang Jiming *et al*. 1990)[7]. Moreover, the prourokinase cDNA was introduced into CHO-dhfr⁻ (Chinese Hamster Overy dihydrofolate reductase-deficient) cells. A new strain which can stably express urokinase-type plasminogen activator (*u*—Pa) was obtained. Our results will be reported in this paper.

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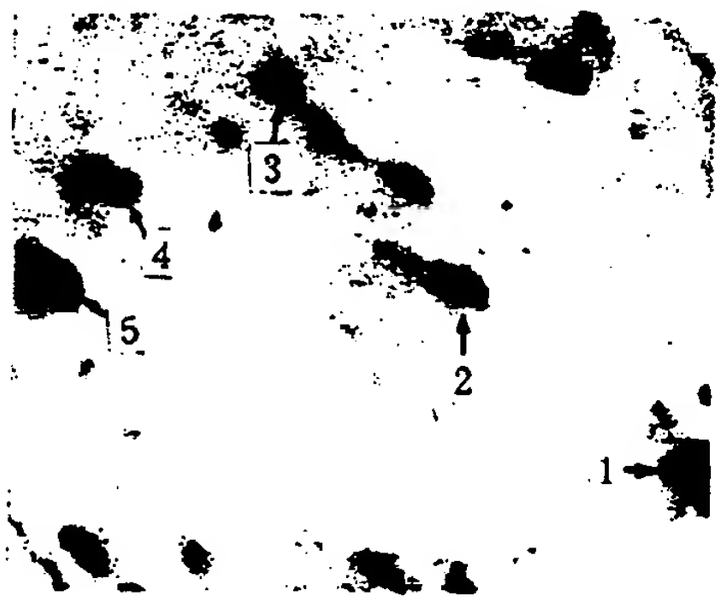


Fig. 2
Hybrydization of recombinant plasmid pSV_2—proUK with ^{32}P labelled pro-UK cDNA probe.
1. Positive control, 2-5. Positive recombinant dots

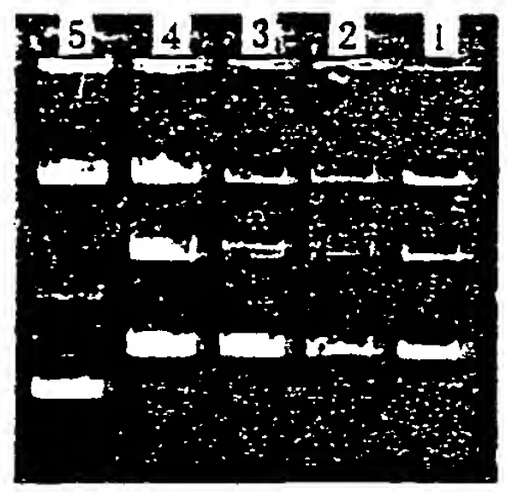


Fig. 3
Analysis of plasmids extracted by boiling method in agarose gel. 1-4. Plasmids from positive colonies; 5. Negtive control plasmid

MATERIALS AND METHODS

The full length cDNA of human prourokinase

The full length cDNA of human pro-urokinase was cloned in our laboratory (Fang Jiming *et al*. 1990) [7].

Bacterial strain and plasmids

RRI (*E. coli*) was conserved in our laboratory; pSV_2-dhfr was obtained from Prof. Ren Guifang; MMTV-dhfr was from Prof. Shen Xiaozhou.

Cells and Media

gth prourokinase cDNA
amino acid residues of
nique and identified by
1990)[7]. Moreover, the
er Overy dihydrofolate
nase-type plasminogen

CHO-dhfr$^-$ cell line was obtained from Prof. Ren Guifang; growth medium (DMEM from GIBCO Co., 10 percent bovine serum, penicillin and streptomycin 100 u/ml, respectively, kanamycin 50 u/ml, L-proline 0.1 m*M*, hypoxanthine 0.03 m*M*, thymine 0.03 m*M*, adjusted pH to 7.2-7.4 with $NaHCO_3$); Selection medium (hypoxanthine and thymine were removed from the growth medium); serum-free selection medium (serum was deleted from the selection medium).

Enzymes and biochemicals

Restriction and other enzymes were purchased from Boehringer Mannheim Co., Sino-American Biotechnology Co., Institute of Basic Medical Sciences of the Chinese Academy of

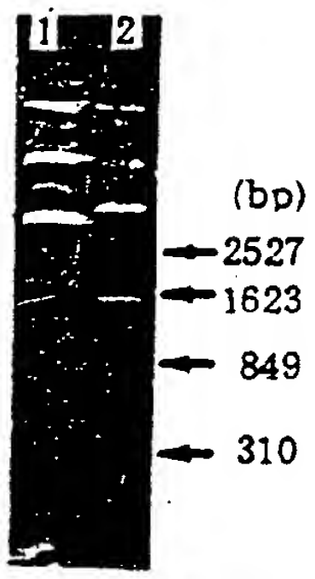


Fig. 4
Identification of pro-UK gene in the plasmid of positive colony by digesting with HidIII and XbaI. 1. pSV_2 -dhfr control; 2. pSV_2-proUK

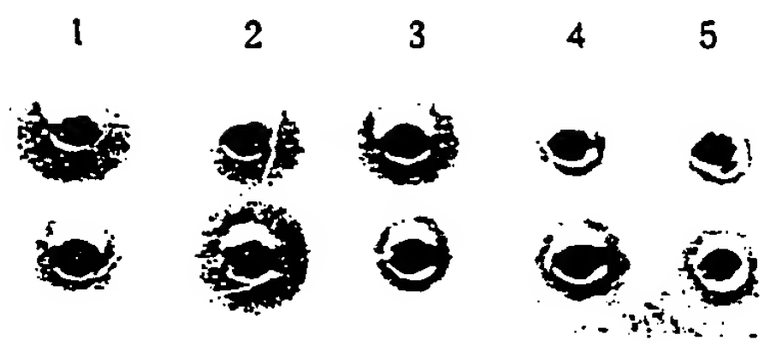


Fig. 5
Expression of pro-UK cDNA in CHO cells by fibrinolytic test of *u*—Pa. Top line 1-4. Standard UK 1.0IU, 0.5IU, 0.2IU, 0.05IU; 5. CHO-dhfr$^-$ supernatant control; Bottom line 1-5. Supernatants of CLF-8, CLF-14, CLF-23, CLF21 and CLF-18

Medical Sciences, and Biolabs Co., [α-^{32}P] dATP (specific activity 6000Ci/mmol) was from Amersham Co.

Standard urokinase, thrombin and fibrinogen

Standard urokinase was purchased from the Chinese Institute for the Control of Pharmaceutical and Biological Products, thrombin from the Factory of Biochemical Products in Tianjin and fibrinogen from Sigma Co.

Anti-serum

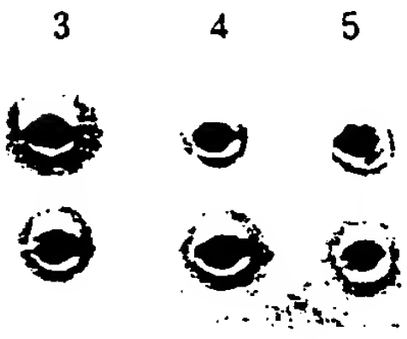
Rabbit against human urokinase anti-serum was prepared in our laboratory (Li Xuzhen *et al.* 1988) [8], rabbit against human tissue-type plasminogen activator (*t*—Pa) anti-serum was obtained from Prof. Ren Wenhua.

Preparations of plasmid DNA

Boiling method for small scale lysis and gentle method for large scale lysis [9,10] were used.

Restriction enzyme digestion, ligation of fragments, transformation, hybridization and identification.

They were carried out according to references [9-11] or with slight modification.



ig. 5
-UK cDNA in CHO
c test of *u*—Pa. Top
d UK 1.0IU, 0.5IU,
; 5. CHO-dhfr⁻
ol; Bottom line 1-5.
f CLF-8, CLF-14,
d CLF-18

Fig. 6
The results of lysis zone test after incubating the fibrinolytic agarose plate at 80°C for 1h 1. 0.5IU standard UK; 2. CLF-14 supernatant 3. 50 BAEE trypsin

Fig. 7
Inhibition of lysis by anti-serum:
1. Standard UK plus UK anti-serum;
2. Standard UK plus *t*—Pa anti-serum;
3. CLF-14 supernatant plus UK anti-serum;
4. CLF-14 supernatant plus *t*—Pa anti-serum.

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nti-serum was obtained

: lysis [9,10] were used.
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Preparation of DNA probe

The specific probe of urokinase DNA was prepared by the Random primer method [12].

Transfection of CHO-dhfr⁻ cell

Both pSV_2-proUK DNA and MMTV-dhfr or pSV_2-dhfr DNA were co-transformed into CHO-dhfr⁻ cells by calcium phosphate precipitation technique according to the procedure reported by Graham *et al.* (1973) [13]. Transformed CHO-dhfr cells were cultured in selection medium, which was renewed in intervals of 3-5 days. CHO-dhfr cell colonies could be seen under the microscope or by naked eyes after 3 weeks.

Test of *u*—Pa activity

The antigenicity of *u*—Pa was determined by ELISA [14] and the biological activity of *u*—Pa was tested by fibrinolytic agarose plate assay [15].

EXPERIMENT RESULTS

Construction and identification of expression vector pSV_2-proUK

pSV_2-dhfr DNA was cut by Bgl II, filled under the catalysis of Klenow fragment, and then digested by Hind III. The 4.3kb fragment of pSV_2-dhfr was recovered from agarose gel while the dhfr fragment was discarded. Pro-UK cDNA was isolated from pMMUK DNA previously digested by HindIII and SmaI. The large fragment of pSV_2-dhfr and pro-UK cDNA were ligated to form the recombinant vector pSV_2-proUK (Fig. 1). *E. coli* RRI competent recipient cells were transformed by pSV_2-proUK. The transformed RRI colonies were replicated by nitro-cellulose filter, and the replic were used for *in situ* hybridization with [α-^{32}P] dATP-labelled DNA probe of prourokinase (Fig. 2). Positive colonies were selected and DNA was extracted. The sizes of extracted plasmids were measured by agarose gel electrophoresis (Fig. 3). The plasmids (pSV_2-proUK) of samples 1-4

Table 1
Concentration of *u*—Pa in serum-free supernatant expressed by CLF-14 and CLF-8.

UK pool	Experiment data						
Standard urokinase	IU	1.00	0.50	0.20	0.10	0.05	0.00
	LZA (mm^2)	113.	93.0	75.0	30.0	8.70	0.00
	ELISA D.	2.04	1.10	0.41	0.20	0.09	0.00
CLF-14	STQ (μl)	30.0	20.0	15.0	10.0	5.00	0.00
	LZA (mm^2)	75.0	-	-	-	-	0.00
	ELISA D.	0.40	0.19	0.18	0.09	0.06	0.00
CLF-8	STQ (μl)	30.0	20.0	15.0	10.0	5.00	0.00
	LZA (mm^2)	44.0	-	-	-	-	0.00
	ELISA D.	0.33	0.17	0.13	0.10	0.05	0.00
Supernatant of CHO cell	STQ (μl)	30.0	30.0	-	-	-	-
	LZA (mm^2)	4.10	4.50	-	-	-	-
	ELISA D.	0.02	0.03	-	-	-	-

Note:

	Cell numbers	Supernatant vol.	Culture time
CLF-14	2.28 × 10	10 ml	48h
CLF-8	2.40 × 10	10 ml	48h
CHO cell	2.85 × 10	10 ml	48h

IU: International unit; STQ: Sample test quantities
LZA: Lysis zone area; ELISA D.: ELISA data (A_{492} values)
Specific activity of urokinase: 10-20 10 IU/mg, i.e.
1 IU equals to 5-10 ng (mean: 7.5 ng)

were obviously larger than that of sample 5 (pSV_2-dhfr). A band of 1.6kb appeared when one of the large plasmids was cut by HindIII and XbaI and analyzed by agarose gel electrophoresis (Fig. 1 and Fig. 4). On the contrary,the 1.6kb cDNA band would not appear if the pro-UK cDNA was inserted in the opposite direction.

Expression of pro-UK cDNA in CHO-dhfr$^-$ cell line

The pSV_2—proUK recombinant plasmid containing SV40 early promoter was cotransfected with MMTV-dhfr (or pSV_2-dhfr) into CHO-dhfr$^-$ cells using the calcium phosphate precipitation technique. The dhfr$^+$ colonies in diameters of 0.5-2.0 mm were picked out after 3 weeks and cultured in 96, 48, 24 well plates and 6 × 4 cm culture vials in succession. Meanwhile, the expression of *u*—Pa was measured by ELISA and fibrinolytic agarose plate assay (serum-free selection medium was used to determine the secretion level of the transfected cells).

Twenty-one out of 66 CHO-dhfr$^+$ colonies were found to express biological activity of *u*—Pa when pSV_2-proUK and pSV_2-dhfr were cotransfected into CHO-dhfr$^-$ cells (PP system); and 92 out of 112 CHO-dhfr$^+$ colonies were found to show *u*—Pa activity while pSV_2-proUK and MMTV-dhfr

0.05	0.00
8.70	0.00
0.09	0.00
5.00	0.00
-	0.00
0.06	0.00
5.00	0.00
-	0.00
0.05	0.00
-	-
-	-
-	-

ure time
48h
48h
48h

ppeared when one of the
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-UK cDNA was inserted

noter was cotransfected
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(PP system); and 92 out
roUK and MMTV-dhfr

were cotransfected into CHO-dhfr$^-$ cells (PM system). The biological activities of *u*—Pa measured by fibrinolytic agarose plate assay showed disparity in expression levels. A colony showing a much higher level of *u*—Pa expression than other colonies was selected from the PP system and named as CLF-14, whose expression level was more than 24IU/10 cells/48h. Another colony from the PM system was also selected and named as CLF-8, whose expression level was more than 16IU/10 cells/48h (Fig. 5 and Table 1).

The supernatants (serum-free) of CLF-14 and CLF-8 also showed strong antigenicity (P/N > 10) in ELISA. The quantity of expressed *u*—Pa from CLF-14 was equivalant to 0.14-0.22 $\mu g/10^6$ cells/48h, and that from CLF-8 was about 0.08-0.14 $\mu g/10^6$ cells/48h (Table 1). Obviously, both the ELISA and fibrinolytic agarose plate assay showed that the products expressed by CLF-14 and CLF-8 possessed not only *u*—Pa antigenicity but also the biological activity of *u*—Pa.

A strange phenomenon was observed in our experiments that some colonies showed strong antigenicity in ELISA but very low or no biological activity of *u*—Pa in the fibrinolytic agarose plate assay. It is suitable for us to select *u*—Pa colonies according to the levels of *u*—Pa biological activity. However, those colonies which showed very low or no biological activity but strong anti-genicity of *u*—Pa would be worthy for theoretical study in the expression and regulation of gene protein products which possess both antigenicity and biological activity.

The following tests were performed to identify that the expression products which caused lysis zones in the fibrinolytic agarose plate assay is *u*—Pa instead of another protease (e.g. trypsin): 1. The ready-made fibrinolytic agarose plate was heated at 80°C for 1h, which inactivated plasminogen. Thus, no lysis zone on the fibrinolytic agarose plate appeared when plasminogen activitor (for example *t*—Pa or *u*—Pa) was added into the wells of the plate. On the contrary, another protease (e.g. trypsin) could still form lysis zones on the inactive fibrinolytic agarose plate. The results of this expriment (Fig. 6) showed that standard urokinase and the supernatant of CLF-14 could not cause lysis zones on the inactived fibrinolytic agarose plate while trypsin still could; 2. Anti-serum neutralization test: Standard urokinase and the supernatant of CLF-14 were mixed with urokinase anti-serum, *t*—Pa anti-serum and nomal rabbit serum, respectively, and incubated at 37°C for 1h. The mixed samples were then added into different wells of the plate. The results indicated that anti-UK serum could completely inhibit the lysis of standard UK and the supernatant of CLF-14 in the fibrinolytic agarose plate assay, while anti-tPA serum and nomal rabbit serum failed to do so (Fig. 7, the result of rabbit nomal serum was not shown). In conclusion, all the above tests proved that a new kind of cell strain secreting active *u*—Pa has been established by genetic engineering.

DISCUSSION

Pro-UK consists of 411 amino acid residues with 12 disulfide bridges and one site of glycosylation. Such a complex protein synthesized in prokaryotic hosts is likely to fold incorrectly or inefficiently, and consequently exhibits low specific activities. Moreover, production of authentic, biologically active prourokinase from cloned pro-UK cDNA requires post-translational modifications such as accurate disulfide bond formation, glycosylation and specific proteolytic cleavage of 20 amino acid signal peptide which are not performed by bacterial cells, but done well in mammalian cells. Some authors[2-6] reported their expriments of pro-UK cDNA in mammalian cells, but the levels of expression were very low. We successfully cloned [7] and expressed pro-UK cDNA in CHO-dhfr$^-$ cells and obtained biologically active *u*—Pa. This result proved that the pro-UK cDNA cloned in our laboratory could function correctly, and the expression level was about 0.07-0.11 pg/cell/24h. We

hope that the expression level of *u*—Pa of CLF-14 cell strain can be further enhanced and developed into a new kind of stable cell line which will be worthy for production by means of subclone selection, copy multiplication of pro-UK cDNA under methotrexate (MTX) stress, improving culture conditions, induction of pro-UK mRNA with phorbol-12-myristate-13-acetase (PMA) and employment of micro-carrier suspension culture in high density.

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